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Extended Summaries SCI Pesticides Group Symposium Research into Bioactive Molecules

The following are extended summaries of papers presented at the meeting Research into Bioactive Molecules, a symposium for Postgraduate Scientists organised by T. Joseph-Horne on behalf of the Physicochemical and Biophysical Panel of the SCI Pesticides Group and held at The Frythe, Welwyn Garden City, Herts., UK on 15 March 1995. They are entirely the responsibility of the authors, and do not necessarily represent the views of the Editorial Board of Pesticide Science.

The Effects of a Synthetic Putrescine Analogue on Germling Development in *Uromyces viciae-fabae*

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Inhibitors of polyamine biosynthesis are now known to control biotrophic fungal pathogens like rusts and powdery mildews both in the glasshouse and in the field.[1,2] Much of this research has centred on the use of enzyme-activated irreversible inhibitors, e.g. α-difluoromethylornithine (DFMO), which is an inhibitor of the putrescine biosynthetic enzyme ornithine decarboxylase (ODC; EC 4.1.1.17). More recently, an alternative method of polyamine perturbation has been demonstrated involving the use of polyamine analogues. Indeed, a variety of such analogues have been found to alter polyamine metabolism in tumour cells, leading to powerful antiproliferative effects.[3] Previous work in this laboratory has shown that the putrescine analogue (*E*)-1,4-diaminobut-2-ene (E-BED) is fungicidal and alters polyamine biosynthesis in fungi grown in vitro.[4,5] It has been suggested that E-BED exerts its fungicidal effects by affecting the early development of the fungus on the leaf surface.[6] This summary reports the effects of E-BED on germination and appressorium formation by uredospores of the rust fungus *Uromyces viciae-fabae* (Pers.) Schroet. on artificial membranes.

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Broad bean plants (*Vicia faba* L. cv. Bunyards Exhibition) were grown in a heated glasshouse until the first leaves were fully formed and were then inoculated with *U. viciae-fabae*, as described previously.[5] Uredospores were collected from infected plants 18 days after inoculation, stored at 4°C until required, and were always used within one month of collection. Immediately before use, uredospores were given a heat shock at 45°C for 3 min and dry uredospores (10 mg) were then dusted onto polyethylene membranes (1·5 cm^2) using a settling tower (30 × 30 × 30 cm). The membranes consisted of plastic bags (200 gauge) scratched with a fine wire brush four times in each of two directions. Each membrane was then floated, spore-side down, in a Petri dish containing either distilled water or inhibitor solution at pH 5·6 (5 ml in each case). After 8 h, the germlings were stained with Coommassie brilliant blue-lactophenol solution and examined under a light microscope (Leitz DM RB with photoautomat, Leica UK). Germination and appressorium formation were assessed by counting 100 germlings in three separate experiments, and data were analysed using Student's *t*-test.

The compound E-BED was synthesised as described previously.[4]

E-BED had little effect on uredospore germination and at 1 mM reduced germination by just 24% ($P < 0·01$); in contrast, appressorium formation was reduced by 37% at 0·05 mM and was completely prevented at 1 mM ($P < 0·01$; Table 1). These data support previous work which showed that this compound was most effective when applied two or three days before or after inoculation, suggesting an effect on early development of the fungus on the leaf surface.[4] The results presented here also agree with recent work showing that

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TABLE 1
Effects of the Putrescine Analogue E-BED on Germination and Appressorium Formation by Uredospores of *U. viciae-fabae* on Artificial Membranes

E-BED concentration (mM)	*Germination (%) (±SED)*	*Appressorium formation (%) (±SED)*
0·00	96 (±0·55)	42 (±3·4)
0·01	94 (±1·5)	39 (±4·2)
0·05	94 (±2·0)	26 (±1·2)
0·1	85 (±0·3)	0·3 (±0·03)
1·0	73 (±1·5)	0

DFMO had no effect on uredospore germination, but a substantial effect on appressorium formation (Reitz, M. & Walters, D. R., unpublished). Although E-BED and derivatives are known to affect polyamine biosynthesis and to reduce intracellular polyamine concentrations, these changes were not considered sufficient to account for their antifungal effects.[4,5] It is important to note however, that work on the effects of putrescine analogues on polyamine biosynthesis were performed on the necrotrophic fungal pathogen *Pyrenophora avenae* S. Ito & Kuribay, while these compounds are most active against biotrophic fungi like rusts and powdery mildews. It would be interesting to determine whether the effect of E-BED on appressorium formation in *U. viciae-fabae*, is associated with a change in polyamine biosynthesis.

ACKNOWLEDGEMENTS

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Molecular Analysis of Azole Fungicide Resistance in a Mutant of *Ustilago maydis*

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The sterol biosynthesis pathway is useful as a fungicide target because differences in the pathway between fungi, plants and mammals allow target specificity. Inhibition of the sterol 14α-demethylation step has been extensively exploited, but resistance to many demethylase-inhibiting fungicides (DMIs) is now a serious agricultural and medical problem. The mechanisms of resistance to DMIs are poorly understood, but some studies with laboratory mutants have implicated changes to the target enzyme, 14α-demethylase (14α-DM). These are mutants of yeast and dimorphic fungi that grow despite apparently defective 14α-DMs, and consequently accumulate high levels of 14α-methyl sterols. One such mutant of *Ustilago maydis* (DC) Corda, ERG40[1] was obtained by *N*-methyl-nitro-*n*-nitrosoguanidine treatment of the wild-type strain ATCC 14826, and selection for polyene resistance using amphotericin-B. ERG40 contains no 14-desmethyl sterols and is clearly blocked at the C-14 demethylation step. However, the molecular basis of this resistance in ERG40 is unclear, although a complete lack of desmethyl sterols indicates that ERG40 is clearly deficient in 14α-DM activity in sterol biosynthesis.

Resistance mechanisms found in laboratory mutants are usually connected with a reduction in overall fitness and consequently are seldom observed in resistant strains recovered from field populations, as they compete poorly with sensitive wild-type strains. If suitable transformation systems are available, it should be possible to identify genes responsible for field resistance by screening for resistance. Isolation and subsequent expression of lanosterol 14α-DM has been achieved in yeasts[2] but application of these molecular techniques to analyse the L14α-DM gene from obligate pathogens such as cereal powdery mildew, is hindered by the lack of any transformation system. Since recombinant DNA technologies are well developed in *U. maydis*, and as ERG40 is clearly deficient in 14α-DM activity, it might be expected to be a suitable recipient for a transformation approach to cloning 14-DM genes from other fungi. Consequently, the main aim of this work was to

determine if ERG40 was a suitable recipient strain for complementation studies.

The L14α-DM gene from *Saccharomyces cerevisiae* Meyer ex Hansen[2] was inserted into the BamH1 cloning-site of the *U. maydis* shuttle vector pUXV1.[3] This construct was transformed into ERG40 by either the sphaeroplast technique[4] or using lithium ions.[5] Transformants were selected on yeast extract potato dextrose (YEPD) plates amended with hygromycin B (200 units ml^{-1}) and were then grown in shake culture in YEPD. Sporidia were harvested, washed and either resuspended for inoculation onto antibiotic- or fungicide-amended YEPD agar assay plates, or freeze-dried for sterol extraction and GC analysis.[6] Hygromycin phosphotransferase (hph) activity was determined by the adaption of a generalised assay for phosphorylation of aminoglycoside antibiotics.[4]

Regardless of the transformation protocol used, it was difficult to obtain transformants consistently from ERG40. The hygromycin B-resistant transformants were clearly altered in terms of their sensitivity to both the DMI propiconazole and the protein synthesis inhibitor cycloheximide (Table 1). All transformants tested had apparently reverted to the wild-type sensitivity of ATCC 14826, and possessed a normal sterol profile. However, similar results were also obtained from ERG40 cells that had been transformed with the vector (pUXV1) alone. Southern analysis[7] was unable to reveal any differences between ATCC 14826, ERG40 and any of the transformants, regardless of the probe used. Hph activity in transformants was negligible (Table 2) in comparison to *Septoria nodorum* (Berk.) Berk. transformants, known to contain the bacterial hygromycin phosphotransferase gene.

Although hygromycin B-resistant transformants of ERG40 with altered DMI sensitivity were apparently obtained, the results from the hph assay clearly show that hygromycin B resistance is due not to the inactivation of the drug by plasmid-determined enzymatic modification but to some other mechanism. This, coupled with the negative results from Southern analysis, indicated that it was possible to complement 14α-DM in ERG40 just by passing cells through the transformation procedure. A final experiment was conducted in which both ERG40 and ATCC 14826 cells were put through the sphaeroplast transformation protocol, and regenerated either on YEPD alone or hygromycin B-amended YEPD. All of the YEPD ERG40-regenerated cells tested had apparently reverted to wild-type sensitivity to DMIs and cycloheximide. Similarly treated cells of ATCC 14826 had remained unchanged in their sensitivity levels, although one strain was now resistant to hygromycin B.

The results of this work strongly suggest that the mechanism of resistance to DMIs in ERG40 is altered by the transformation procedure. Consequently, ERG40 is not a suitable recipient host for the cloning and expression of 14α-DM genes.

TABLE 1
Fungicide and Antibiotic Minimum Inhibitory Concentrations for ERG40, ATCC 14826 and Transformants[a]

Test strain/mutant	*Hygromycin B*	*Propiconazole*	*Cycloheximide*
ERG40	<25	>5	0·01
E-T8[b]	>200	0·62	1
E-T9[b]	>200	0·62	>1
E-TA[b]	>200	0·62	>1
ATCC 14826	50	0·62	0·1
E-UX-1[c]	>200	0·62	>1
E-UX-6[c]	>200	0·62	1
E-UX-7[c]	>200	0·62	1

[a] All values are μg ml^{-1}, except units ml^{-1} for hygromycin B.
[b] T = transformed with pUXV1 + S.c. L14 DM gene.
[c] UX = transformed with pUXV1.

TABLE 2
Summary of Results of Hygromycin-B Phosphotransferase Assays

Test strain/mutant		*Dpm mg^{-1} protein × 10^3*
Septoria nodorum		
Sn171/T1	Transformant	98·7
Sn171/T2	Transformant	70·2
Bs171	Wild-type	8·95
Ustilago maydis		
ERG40	Mutant	NONE
E-T8	Transformant	2·5
E-UX-4	Transformant	NONE
A1	Transformant	NONE
AV1	Transformant	5·5
AV2	Transformant	4·2
E3	Transformant	2·7
E54-TFC-9	Transformant	2·7

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The Use of Artificial Neural Networks to Model Plant–Environment Interactions

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Many environmental systems are complex, and are influenced by interactions between a large number of variables. Artificial Neural Networks are excellent tools for analysing and interpreting these interactions, as they have the ability to model nonlinear functions and can cope with complex 'noisy' data. One example of a complex environmental system is the influence that leaf age and microclimate have on the dose response of clover (*Trifolium subterraneum* L.) to the pollutant, ozone.[1] Ozone is a secondary pollutant commonly occurring in episodes of around 100 nl litre^{-1} in the United Kingdom. It is highly oxidising in nature and readily damages plant tissues, causing a chlorotic flecking on the leaves.

In order to study ozone microclimate interactions, greenhouse-grown clover plants were exposed to a range of ozone concentrations between 0 and 160 nl litre^{-1} (10 am to 5 pm) using a closed-chamber exposure system.[1] Photosynthetically active radiation (PAR), temperature, relative humidity (RH) and ozone concentrations were measured during each ozone exposure, and logged onto a personal computer. After exposure, the plants were returned to the greenhouse for one week, after which visible ozone injury was assessed on the first three leaves, using an injury score key ranging from 0 to 5, where 0 represents no injury and 5 represents total injury cover of the leaf.

Results from these exposures indicated that ozone injury symptoms developed on clover leaves at AOT40 values (the dose accumulated above a threshold of 40 nl litre^{-1} during daylight hours)[2,3] greater than *c.* 200 nl litre^{-1} h. This visible injury increased on the first leaf to a mean score of 4 at an AOT40 of around 750 nl litre^{-1} h (Fig. 1). However, this dose–response showed a great deal of scatter, largely due to interactions between ozone and microclimate.

These interactions were investigated further by the use of a Back Propagation Artificial Neural Network. This consisted of three layers of nodes: an input layer representing the causal agents, a hidden feature detection layer, and an output layer representing the effects of the causal agents. These layers were interconnected by weighted links (essentially mathematical functions which modify input values to produce output values; Fig. 2). Six input nodes representing light, temperature, relative humidity (RH), vapour pressure deficit (VPD), ozone dose AOT40 and leaf age were used, along with 12 hidden nodes and one output node representing the mean visible injury score. The network was trained on data from the ozone exposure experiments. The Neural Network looked for patterns in these data by modifying weightings of the interconnections in response to a comparison with the data. Testing of the network was carried out during the training process by repeatedly presenting it with inputs not previously seen. Comparison was made of the injury predictions for the test inputs with the injury which actually occurred. Training was considered complete when the error in these predictions stopped decreasing consistently.

The weightings of the network were analysed to give a relative importance of the various input parameters (Fig. 3). As expected, the strongest influence was clearly

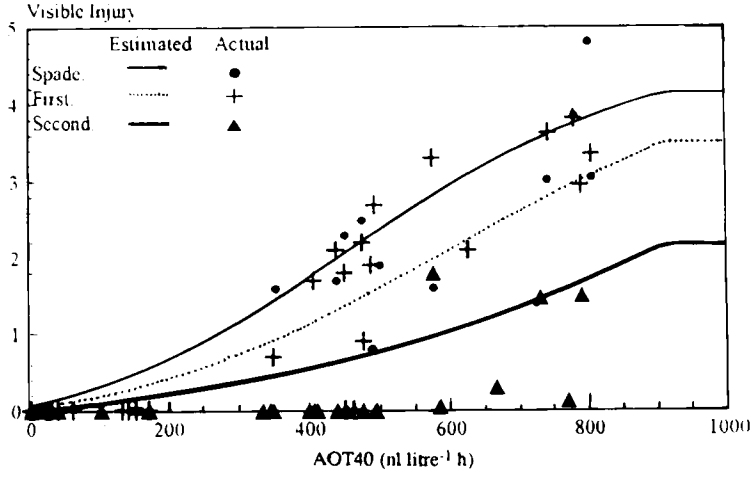

Fig. 1. Visible injury response in *Trifolium subterraneum*.

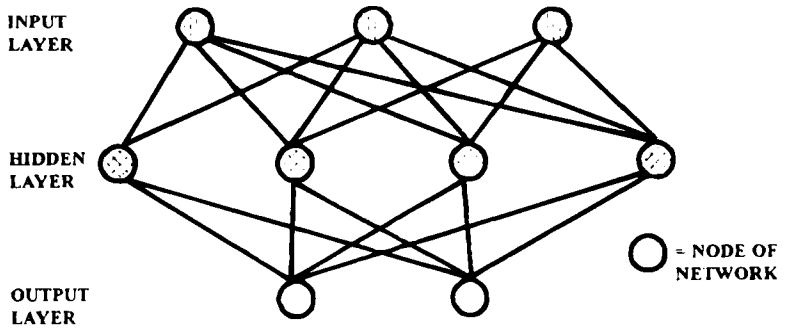

Fig. 2. Neural Network Structure.

AOT40, since this was the factor which caused the injury. Relative humidity was the next most important factor. This is known to have a strong influence upon the functioning of the stomata, affecting the amount of ozone flux into the leaf, and thus the extent of injury. Leaf age, which was likely to be a function of the physiological state of the leaves and their stomata had a similar but slightly lower weighting.[4] Light had the next strongest influence, possibly due to its effect on relative humidity; vapour pressure deficit and temperature exerted weaker influences.

To gain a further insight into the interactions between leaf age, ozone dose, and visible injury, the trained neural network was presented with a range of ozone doses for the spade, first and second leaves. The microclimate inputs were kept constant at 45% relative humidity, 21°C temperature, 150 μmol photons m^{-2} s^{-1} light and 1·4 kPa VPD. The resulting output produced a predicted dose response for the three leaves and these predictions were compared with the actual data (Fig. 4).

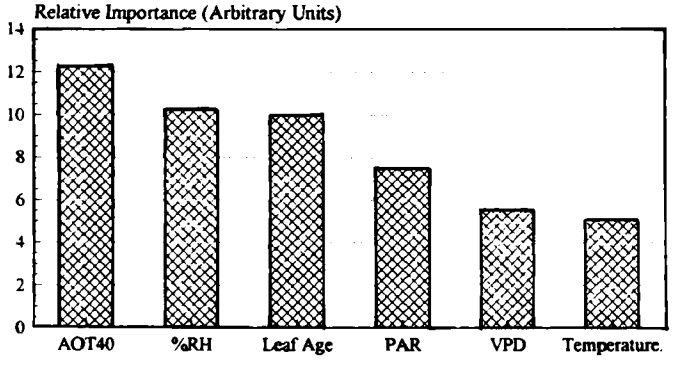

Fig. 3. Relative importance of input parameters of the trained neural network.

Predicted dose response curves for the spade, first and second leaves showed that the spade leaf (a true leaf, but not the first true trifoliate leaf) is the most sensitive, followed by the first leaf, then the second. Predictions of injury for the three leaves show a reasonable fit to the actual data, despite a lot of scatter. It has been reported that sensitivity increases with leaf age.[4]

Neural Networks are clearly a useful tool for modelling and interpreting the complex data found in environmental systems. Analysis of neural network models can indicate the important influences in the environmental system concerned, as well as produce a predictive model of the system under a wide range of conditions. Such systems could include plant/environment growth models, optimisation of spray time, pest/disease identification and herbicide efficacy/environmental interactions. This work is discussed in greater detail in a forthcoming publication.[1]

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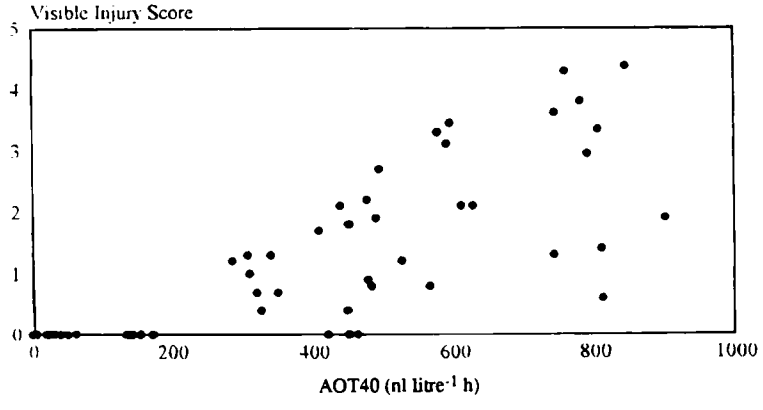

Fig. 4. Actual and neural network predicted injury for differing leaf ages under constant microclimatic conditions.

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Continuum Regression: Optimised Prediction of Biological Activity

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Studies of Quantitative Structure–Activity Relationships (QSAR) aim to relate the biological activities of drugs and agrochemicals to their physicochemical properties by way of mathematical techniques. The most popular methods include Multiple Linear Regression (MLR), and the 'latent variable' techniques Partial Least Squares Regression (PLS) and Principal Components Regression (PCR). The goal is to determine a QSAR that is predictively powerful, i.e. one that yields accurate and robust predictions of future compounds.

There are two problems that have to be considered when constructing QSARs. First, how should the QSAR be constructed, i.e. which method should be used? Each method has its own set of assumptions about the data, and if these assumptions are not satisfied the analysis may be prone to problems. The second problem is how to define the QSAR, or how may variables (original or latent) should be included in the model? Cross-validation[1] is regularly employed to define a model, but this method is often misused and can lead to over-confidence in the model. This summary reports a new technique called Continuum Regression (CR) for constructing predictively powerful QSARs.

Continuum Regression is a generalised regression technique which allows latent variables to be constructed according to different criteria, e.g. to maximise the correlation, the covariance or variance by introducing an adjustable parameter, α. CR, reformulated at Portsmouth[2,3] from the original proposal,[4] aims to overcome deficiences in computational efficiency and lack of mathematical rigour. The Portsmouth CR algorithm strives to maximise the Generalised Criterion Function (GCF)

$$T = (\mathbf{c}'\mathbf{X}'\mathbf{y})^{(2+2\alpha-4\alpha^2)}(\mathbf{c}'\mathbf{X}'\mathbf{X}\mathbf{c})^{(-1+2\alpha)} \quad (1)$$

where $\mathbf{X}$ is the matrix of physicochemical properties, $\mathbf{y}$ is the response variable and $\mathbf{c}$ is the vector of component loadings.

While in the original formulation α was given a range $[0, 1]$,[4] the new formulation allows α to take any value. Adjusting α allows CR to produce analyses equivalent to MLR ($\alpha = 0$), PLS ($\alpha = 0.5$) and PCR ($\alpha = 1$) and also a compromise between MLR and PLS or PLS and PCR (see Fig. 1). The Portsmouth CR formulation also allows for an analytically optimal solution of α to be determined by the algorithm.

Model specification

The number of latent variables in the final model can be determined by Leave-One-Out (LOO) cross-validation using the I statistic[4] defined as

$$I = 1 - \frac{PRESS_i}{PRESS_{no}} \quad (2)$$

where $PRESS$ is the predictive residual sum of squares, i is the number of latent variables in the model and $PRESS_{no}$ is based on the mean value of the response. The I statistic is equivalent to a coefficient of determination for prediction; I is the proportion of the total prediction sum of squares explained.

Example—Solubility properties in biological media: Kidney-gas partition coefficients

This example is based on the work of Kamlet *et al.*[5] The data set was analysed using MLR, PLS, PCR and the optimised α option using an in-house CR algorithm written in FORTRAN 77 and run on a VAX 6310 together with models specified by the number of latent variables yielding the highest I statistic, and the equivalent model R^2 is also reported. The 'pseudo-beta' coefficients are the regression coefficients of the original variables that each model yields.

These authors related the distribution, redistribution and elimination of xenobiotics to their solubility in and

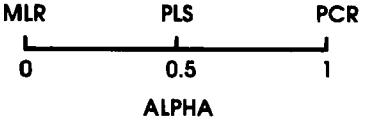

Fig. 1. Diagrammatic illustration of the range of α.

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TABLE 1
Summary Results of the MLR, PLS, PCR and CR Analyses of Kidney-Gas Partition Coefficients

				Pseudo betas		
Method	α	*max I*	*no LVs*[a]	$V_1/100$[b]	π^*[c]	β_m[d]
MLR	0·0	58·39	1	−3·699	−1·274	−3·504
PLS	0·5	57·74	2	−2·164	−1·149	3·914
PCR	1·0	57·99	3	−3·699	−1·274	3.504
CR	1·60	73·21	1	−3·069	−0·905	3·605

[a] Latent Variables.
[b] $V_1/100$ is the intrinsic (Van der Waals) molar volume.
[c] π^* is the solute dipolarity/polarizability parameter.
[d] β_m is the hydrogen bond basicity parameter.

partition between the kidney and gas phases. Table 1 shows that the CR model is significantly better for prediction purposes than those produced by MLR, PLS and PCR. Note that α is outside the range [0, 1].

Degenerate data sets

A problem inherent in both CR algorithms is that it cannot deal with degenerate data sets, i.e. when one or more descriptor variables do not contribute any unique information. This is a common problem in QSAR and a solution is to reduce the number of variables in the data set. The problem is how to eliminate redundant variables without reducing the amount of information explained by the response variable.

The data technique (Whitley, D., pers. commun.) is based upon the multiple correlation coefficients of each variable with all other variables. By finding the lowest pairwise correlation coefficient between variables, the two most uncorrelated variables are identified. Then other variables are added to the data set according to the lowest multiple correlation coefficient until all coefficients then calculated are unity. This technique has the advantage of eliminating redundant variables whilst maintaining all of the information explained in the response variable.

TABLE 2
Cumulative Percentage Response Explained by PLS Analysis of Syzdlo's Full Data Set and PLS, PCR and CR Analysis of the Reduced Data Set

		Reduced data set		
LV No.	*Full set PLS*	*PLS*	*PCR*	*CR* $\alpha = 0{\cdot}65$
1	31·7	17·96	23·47	14·90
2	38·9	34·76	31·10	32·52
3	41·7	36·92	32·52	34·55
4	45·4	38·35	33·31	36·34
5	49·6	46·91	33·98	45·29
6	51·8	49·92	43·11	48·29
7	51·8	51·59	50·67	51·51
8	51·8	51·68	51·67	51·68
9	51·8	51·76	51·76	51·76

Example—Toxicity of pyrethroid insecticides

The data set of Syzdlo[6] contained 15 descriptor variables measured on 16 observations, and contained seven redundant variables (identified by preliminary Principal Components Analysis (PCA)). The data reduction technique identified that nine original variables could adequately explain all of the information in the response. The analysis shows that the PLS analysis of the full data set is matched by the subsequent analyses using the CR algorithm on the reduced data set. The analyses are summarised in Table 2, where the percentage response explained by each model is given for PLS on the full data set and PLS, PCR and CR on the reduced data set. The results show that the fits of the models are not very high (R^2 = 51·8%). The prediction rates (I) for all the models are very low ($I < 0$) indicating the poor quality of the data set for prediction purposes.

Thus, Portsmouth formulation of CR offers an important analytical technique that provides QSAR that are always as good as and sometimes better than those produced by MLR, PLS and PCR for prediction of novel chemical structures. The Whitley data reduction technique has been shown to provide a smaller number of variables without losing any of the information in the response, so enhancing the interpretability of the final QSAR.

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An Investigation into Chlorotoluron Resistance in Blackgrass (*Alopecurus myosuroides*)

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Blackgrass (*Alopecurus myosuroides* Huds.) is a major weed in the UK, particularly in winter-sown cereal crops. The extensive use of the herbicide chlorotoluron (CTU) has led to the development of resistant blackgrass populations, which was first reported in 1984.[1] Populations exhibit varying degrees of resistance and have been categorised; the most resistant to date was in Peldon, Essex, UK.[2] There is no difference between biotypes in their uptake and translocation of the herbicide, but metabolism of CTU occurs two to three times faster in resistant than in susceptible biotypes.[3] There is also evidence that this enhanced metabolism is carried out by cytochrome P450 monooxygenases, and this is presumed to form the basis of resistance.[4] The object of this study was to investigate the resistance of a blackgrass population from Peldon using the parameters of growth and photosynthesis in whole plants.

Susceptible (Herbiseed Ltd, Berkshire, UK) and resistant (Peldon, Essex, UK) blackgrass seeds were sown in 7-cm pots containing J Arthur Bowers potting compost under glasshouse conditions (20/15(±5)°C) and thinned to six plants per pot. Chlorotoluron 500 g litre^{-1} SC (Dicurane 500FW) was applied at the two- to three-leaf stage at rates ranging from 0.44 to 49 kg AI ha^{-1} using a Mardrive evaluation unit set to deliver 200 litres ha^{-1} (fixed 80° flat fan nozzle, 3 bar pressure). Plants were harvested two weeks after spraying and the ED_{50} value, the rate required to reduce fresh weight by 50%, was determined. Gaseous exchange was measured using an infra-red gas analyser (IRGA, ADC, Herts, UK). For each experiment six plants in individual pots (35 mm) were used; three were sprayed with CTU at field rate (3·5 kg AI ha^{-1}) and three with distilled water. Each plant was then put into a sealed, purpose-built, acrylic chamber (160 × 40 × 40 mm) through which ambient air flowed continuously at 400 ml min^{-1}. The chambers were connected to the IRGA via a gas handling device which allowed readings to be taken sequentially from each chamber every 5 min. The plants were monitored for carbon dioxide uptake in continuous light (300 μmol m^2 s^{-1}) at 23°C for 24 h after spraying.

The data presented in Fig. 1 show the rate of decline of fresh weight when plants were sprayed with increasing chlorotoluron rate and clearly demonstrate the extent of the resistance. Susceptible plants sprayed with CTU at field rate showed leaf chlorosis, and the production of new leaves and tillers was severely reduced. The control plants for both biotypes had similar fresh weight values, indicating that resistance was not associated with a reduction in plant growth. The ED_{50} values, based on fresh weight analysis two weeks after spraying,

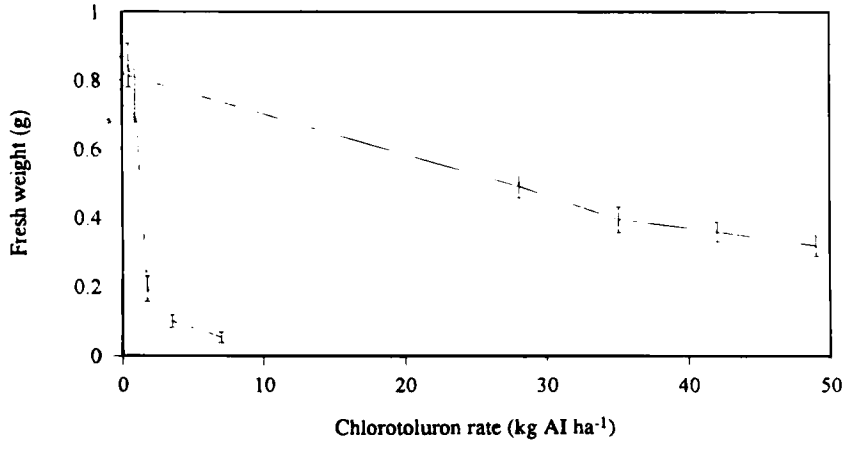

Fig. 1. The reduction in fresh weight of (——) susceptible and (– – –) resistant blackgrass when sprayed with increasing chlorotoluron dose. Values represent means ± SE, $n = 30$.

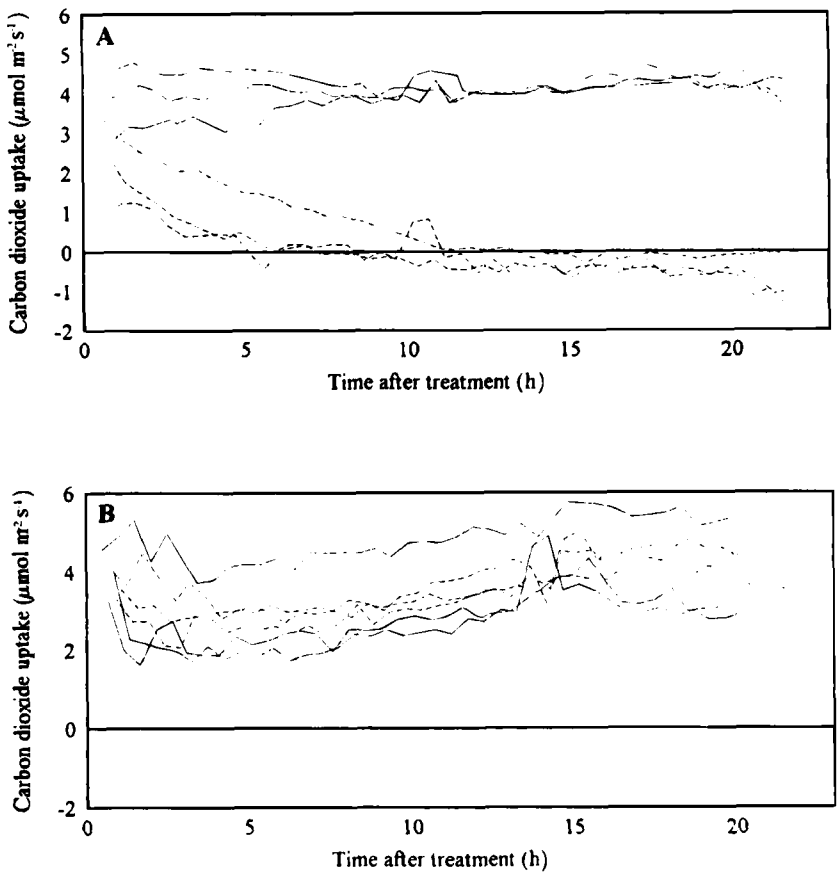

Fig. 2. Photosynthesis by blackgrass plants after they were sprayed with (——) water or (– – – –) chlorotoluron (3.5 kg AI ha^{-1}); (A) susceptible and (B) resistant plants. Each trace represents the response of a single plant.

were calculated to be 1·05 and 36·5 kg AI ha^{-1} for susceptible and resistant plants respectively, giving a resistance factor of 35. The ED_{50} value given here for the Peldon population is similar to that reported elsewhere from simulated field conditions.[5] The calculation of resistance factors naturally depends on the control biotype and the ED_{50} value presented here concurs with those reported for the standard susceptible biotype from Rothamsted, UK.[6]

Figure 2(a) displays the rate of photosynthesis over a 24-h period by individual susceptible plants, in the presence and absence of CTU. Photosynthesis rates declined steadily to zero in treated plants, ceasing approximately 12 h after spraying. However, photosynthesis by control plants remained constant or increased slightly over this period, indicating that the decline in treated plants was not due to stress as a result of being enclosed in the chambers. The rate of photosynthesis by resistant plants is shown in Fig. 2(b); treated plants did not show any decline with time, and rates were similar to those in control plants. Photosynthesis increased marginally in all plants with time, which was probably due to the small increase in leaf area expected with actively growing plants.

In conclusion, we have demonstrated that blackgrass from Peldon has a resistance factor of 35, and that field rate CTU does not suppress its photosynthetic rate. Further studies are being carried out to measure several other growth and physiological parameters with time after spraying. Once characterisation of resistance is complete, it will form the basis of further investigations into resistance mechanisms.

ACKNOWLEDGEMENTS

We thank Dr S. Moss for organising the collections of Peldon seed and the Higher Education Funding Council for England for financial support.

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A Study of Antagonism Between the Herbicide Phenmedipham and Ozone Pollution in Sugarbeet

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Spring-sown crops, such as sugarbeet, are sprayed with early post-emergent herbicides to control the growth of spring-germinating weeds. However, spraying can coincide with ozone episodes, typically of two to three days duration, when concentrations of between 50 and 90 nl ozone litre^{-1} of air occur for about 5 h per day.[1] These episodes occur in Britain in spring and summer and may influence the safety of the herbicide on the crop, resulting in increased crop damage and decreased crop yields. In this study, the possibility of an interaction occurring when the photosynthetic inhibitor herbicide phenmedipham is applied shortly after an ozone episode was investigated. Both ozone and phenmedipham are known to increase the production of active oxygen species and hence are likely to alter the activities of the endogenous scavenging system. Phenmedipham blocks the electron-transport chain, leading to the breakdown of carotenoids due to excess electrons which, in turn, increases the singlet oxygen concentration within the chloroplast. In contrast, ozone reacts with water molecules at the surface of the cell membranes, producing superoxide, hydrogen peroxide and singlet oxygen.

In each experiment, sugarbeet plants (*Beta vulgaris* L. cv. Saxon) with two to three leaves were exposed to a simulated two-day ozone episode (100 nl litre^{-1}, 7 h per day) in a closed exposure system.[2] Three days later, the plants were sprayed with a phenmedipham 114 g litre^{-1} EC ('Betanal' E; AgrEvo, UK, Ltd) at the field rate of 1·14 kg AI ha^{-1}.

In an initial investigation, shoot fresh weights were determined seven days after herbicide treatment. Results indicated that a less than additive interaction was occurring (Fig. 1), possibly suggesting that either ozone or phenmedipham, or both, were not exerting their full effect. The expected additive values were the sum of the reductions attributable to ozone alone and phenmedipham alone subtracted from those for untreated plants.

To characterise this interaction further, samples of leaf tissue (1 g) were taken at daily intervals after the end of ozone exposure from eight plants per treatment, for eight days. These were extracted and assayed for the enzymes glutathione reductase (GTR; E.C. 1.6.4.2) and glutathione-*S*-transferase (GST; E.C. 2.5.1.18) and the antioxidant, glutathione. The extraction procedure was undertaken according to Hull,[3] yielding a supernatant, a portion of which (2·5 ml) was desalted by passing it through a Sephadex G-25 PD-10 column prior to determination of GTR[4] and GST[5] activities and protein content.[6] The remaining undesalted supernatant (2·5 ml) was used to determine total glutathione content of the tissue using a modification of the method of Griffith.[7] Enzyme activities were expressed on a protein basis.

Protein contents of plants treated with ozone alone and with ozone and phenmedipham were decreased by 30 and 15% respectively compared to control values (Table 1). This diminution has also been observed in barley treated with ozone,[8] but other studies have shown no reductions in soluble protein contents of leaves exposed to ozone at comparable concentrations.[9] Ozone affects several amino acids, including opening the pyrrole ring of tryptophan and oxidising the sulphydryl groups of cysteine and methionine.[10]

In this study, a large decrease in total glutathione content was observed in response to ozone alone and phenmedipham alone three and two days respectively after treatment (Table 1). Concurrent increases were shown in the activities of both GTR and GST in response to ozone exposure. Data for the other antioxidant-scavenging enzymes, including superoxide dismutase, ascorbate peroxidase, catalase and non-

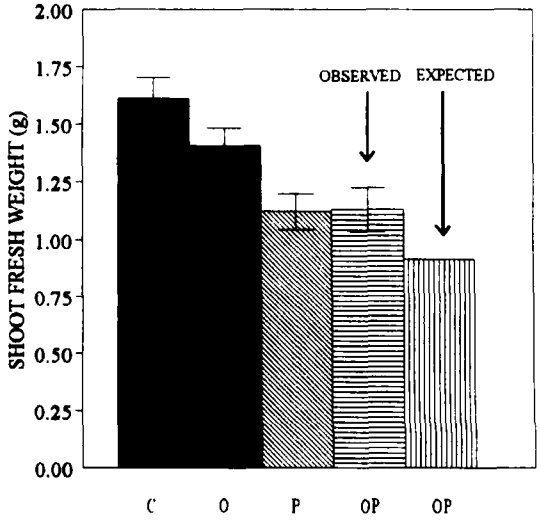

Fig. 1. Effect of ozone and/or phenmedipham on sugarbeet (*Beta Vulgaris* cv. Saxon) shoot fresh weight. Values are means ± standard error where $n = 4$. (C) controls, (O) ozone, (P) phenmedipham, (OP) ozone and phenmedipham.

TABLE 1
The Effects of Ozone and/or Phenmedipham on the Protein and Total Glutathione Content and Activities of Glutathione Reductase and Glutathione-*S*-transferase[a]

Substance	*One day after ozone exposure, ozone alone*	*Immediately before spraying, ozone alone*	*Two-days after spraying (Five days after ozone exposure)*		
			Ozone alone	*Herbicide alone*	*Ozone + herbicide*
Protein	89	70	102	107	85
Total glutathione	95	49	42	37	39
Glutathione reductase	143	141	108	143	192
Glutathione-*S*-transferase	144	172	113	132	162

[a] Values are percentage of the control means, where $n = 8$. Means of untreated controls over all three days: Protein 118·6 mg per two leaves; Total glutathione 395·6 mg g^{-1} fresh weight; Glutathione reductase 48·8 nmoles mg^{-1} protein min^{-1}; Glutathione-*S*-transferase 20·9 nmoles mg^{-1} protein min^{-1}.

specific peroxidases, will be described elsewhere.[2] Similar increases in the activities of these enzymes have been shown in ozone-treated barley to correlate with a decrease in the total glutathione pool.[8] In addition, a 26-fold increase in GST mRNA was observed in *Arabidopsis thalina* (L.) Heyhn. within 3 h of exposure to ozone.[11] These observations would indicate that an increase in GST activity is an important response to ozone, although it is not clear whether it occurs through conjugation of toxins or as a result of peroxidase activity.[8] Phenmedipham increased GTR and GST activities by 40% immediately after treatment and similarly reduced the total glutathione content by 50%. Plants treated with ozone followed by phenmedipham increased GTR and GST activities by 90 and 50% respectively (Table 1), indicating an additive response in these particular enzymes. However, total glutathione content (Table 1) decreased, in a less than additive way. One possible explanation is that less conjugation is occurring in plants treated with ozone and phenmedipham, and therefore that GST may have a more important role as a peroxidase. Thus the stimulation of some of the antioxidant enzymes is likely to be of key importance in the interaction described in this summary.

ACKNOWLEDGEMENTS

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Involvement of Human Cytochrome P450 3A4 in the Metabolism of Vamidothion

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Vamidothion [*O,O*-dimethyl *S*-2-(1-methylcarbamoylethylthio)ethyl phosphorothioate] (1, Fig. 1) is a widely used organophosphorus insecticide and plays an important role in the control of sap-sucking insects and mites feeding on fruit trees, plants and crops. Toxicity of vamidothion has been observed in mammals and insects, causing nerve poisoning by inhibiting cholinesterases (ChEs)[1,2] and prolonged exposure to the material is reported to induce a genetic effect in factory workers.[3] Metabolism of vamidothion has been studied in insects, mammals and plants.[4] In-vivo and in-vitro metabolism studies in house fly[5] and rat[6] have shown vamidothion to be rapidly oxidized by cytochrome P450 to the sulfoxide (2, Fig. 1), sulfone and into water-soluble metabolites as a result of cleavage of P—S and S—C bonds.

Cytochrome P450s are members of a superfamily of monooxygenases which are widely distributed throughout animal, plant and microbial systems.[7] Human cytochrome P450 3A4 is the major form in the liver and has been shown to be involved in the oxidation of drugs,[8–10] steroids,[11] and environmental pollutants.[12] However, its role in the metabolism of pesticides remains unclear. Heterologous expression of cytochrome P450s in yeast provides a useful tool for studying pharmacological and toxicological aspects of individual enzymes.[9] Using a yeast expression system we have studied the role of human cytochrome P450 3A4 in the metabolism of vamidothion using whole cells and microsomal fractions.

Human cytochrome P450 3A4 cDNA was expressed using the vector pAAH5 in *Saccharomyces cerevisiae*

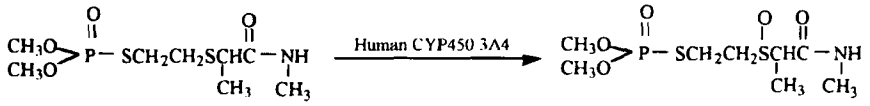

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Fig. 1. Metabolism of vamidothion by human cytochrome P450 3A4. 1, Vamidothion. 2. Vamidothion sulfoxide.

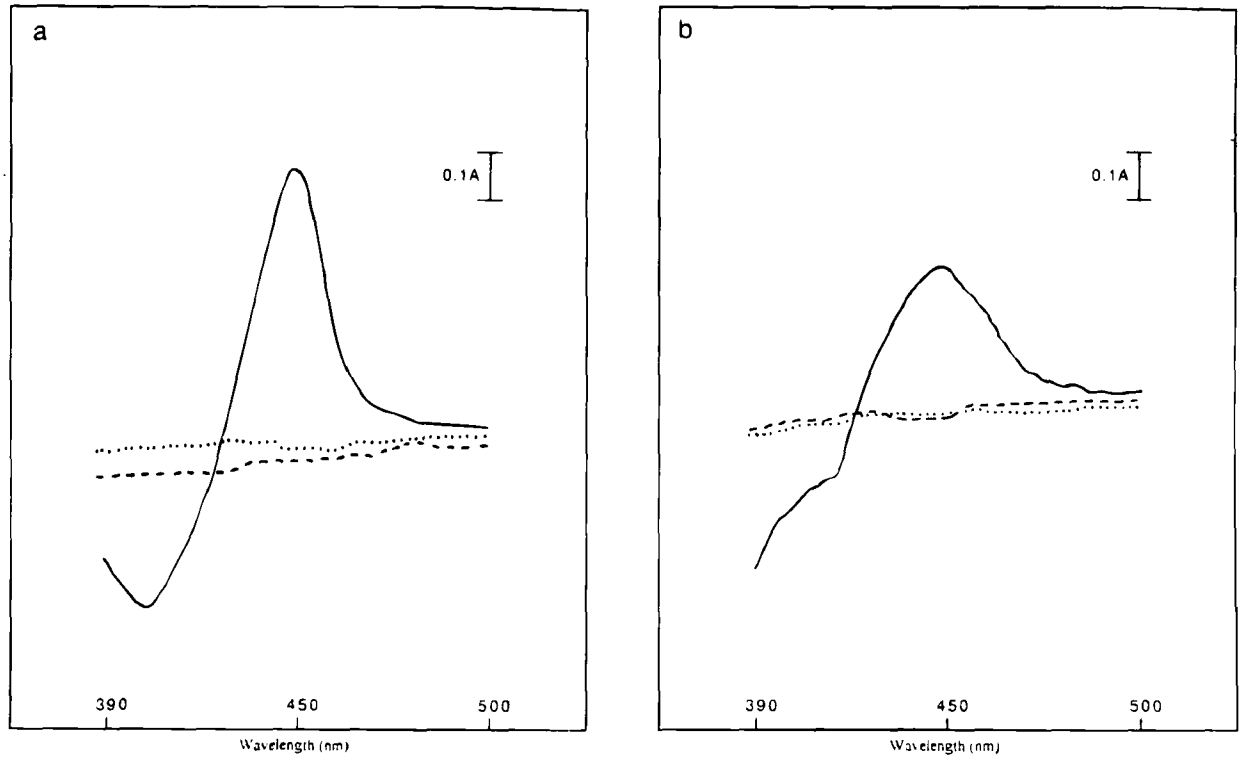

Fig. 2. Reduced carbon monoxide difference spctrum of (a) microsomal fractions and (b) whole cells of yeast expressing human cytochrome P450 3A4. Yeast microsomal fractions were prepared as described previously,[13] using buffer pH 7·4 containing glycerol (200 ml litre^{-1}), potassium phosphate (0·2 M) EDTA (1 mM) at a protein concentration of 2 mg ml^{-1}. A few grains of sodium dithionite were added and the sample divided between two cuvettes. The baseline was recorded (·····), carbon monoxide was bubbled into the sample cuvette and the difference spectrum was recorded (———) between 390 and 500 nm. The measurement was repeated with control yeast microsomes prepared from transformants containing pAAH5 (– – –). Similar spectra were also obtained from cells transformed with pAAH5 containing cytochrome P450 3A4 cDNA (———) and pAAH5 (– – –) after suspension in potassium phosphate buffer (0·2 M; pH 7·4) at the concentration of 5 × 10^7 cells ml^{-1}.

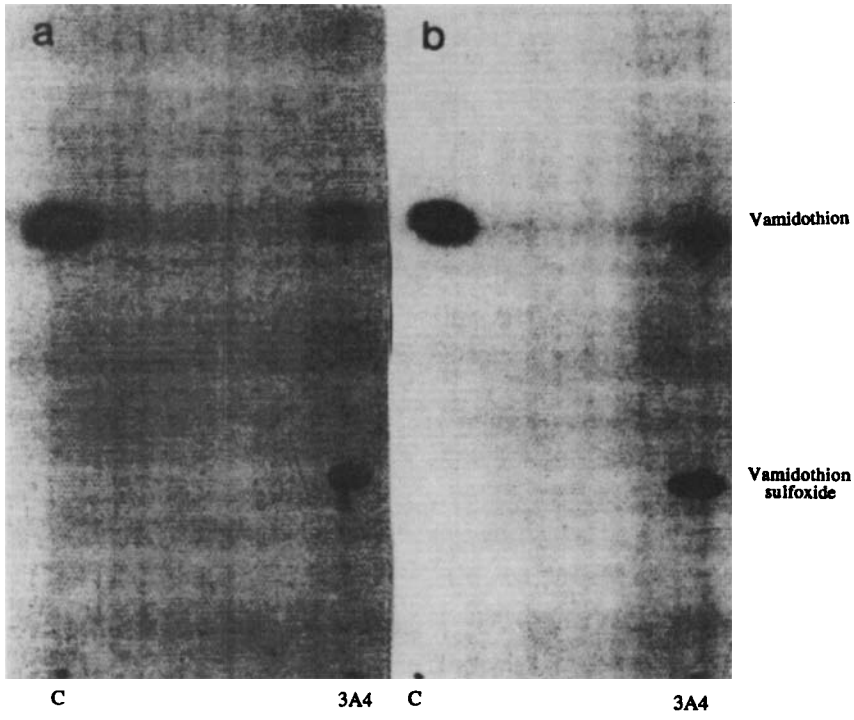

Fig. 3. TLC analysis (autoradiography) of [^{14}C]vamidothion metabolized by human cytochrome P450 3A4. (a) In-vivo biotransformation, (b) In-vitro biotransformation. C: Organic extract from control yeast containing pAAH5 alone, spotted on TLC. 3A4: Organic extract from yeast expressing human cytochrome P450 3A4. *In-vivo biotransformation:* ^{14}C-labelled vamidothion 1·2 μCi (7·18 μmol) was added to yeast cultures. After 40 h incubation at 30°C, when cells reached their early stationary phase, the metabolites were extracted, dried under nitrogen and resuspended in ethyl acetate. *In-vitro biotransformation:* Yeast microsomal fractions containing 300 pmole P450 3A4 and an equivalent concentration of microsomal protein from control yeast were preincubated with 0·5 μCi (3 μmol) [^{14}C]vamidothion at 37°C for 3 min in buffer containing glycerol (200 ml litre^{-1}) potassium phosphate (0·2 M), EDTA (1 mM); pH 7·4. The reaction was initiated by the addition of NADPH (1 mM) in a final volume of 1 ml. The reaction was terminated after 30 min and metabolites extracted using ethyl acetate. Metabolites were identified by TLC with reference to a known standard in benzene + chloroform (55 + 45 by volume). Autoradiographs were developed and metabolites were quantitated by scintillation counting. The R_f values observed for vamidothion and vamidothion sulfoxide were 0·66 and 0·25 respectively.

Meyer ex Hanson strain AH22. Transformants were grown and maintained at 30°C on minimal medium.[11] Microsomal fractions were prepared and the P450 level was estimated by reduced carbon monoxide difference spectroscopy.[13] Protein content was determined as reported previously.[11]

In the reduced carbon monoxide difference spectrum, expressed cytochrome P450 3A4 haemoprotein showed a typical cytochrome P450 peak at 448 nm in both microsomal fractions and whole cells (Fig. 2). A specific content of 175(±15) pmoles cytochrome P450 3A4 mg^{-1} microsomal protein was observed and a yield of 1·25(±0·25) nmol litre^{-1} of culture was obtained. Control transformants containing pAAH5 produced microsomal fractions and cells with undetectable yeast P450 levels determined by reduced carbon monoxide difference spectra (Fig. 2).

Metabolism of ^{14}C-labelled vamidothion was observed in transformed yeast. In in-vivo biotransformation studies, vamidothion was incubated with transformants expressing human cytochrome P450 3A4. When cells reached early stationary phase, metabolites were extracted using ethyl acetate, identified by TLC (Fig. 3) and quantified by scintillation counting as described previously.[8] After 40 h incubation, 65% of vamidothion had been oxidized into vamidothion sulfoxide (Fig. 4) as indicated by TLC R_f values (Fig. 3) and results of scintillation counting. This biotransformation has been reported previously[14] in mammalian liver, and the toxicity of vamidothion sulfoxide has been observed to be lower than that of vamidothion in insects and animals.

Use of microsomal fractions also confirmed the role of human cytochrome P450 3A4 in oxidation of vamidothion to its sulfoxide metabolite and 73% conversion was found during 30 min incubation for cellular metabolism (Fig. 4). This level of conversion is in agreement with previous work with mouse liver.[6] However, in our findings, vamidothion was oxidized only to the sulfoxide and we did not detect the sulfone or other water-soluble metabolites. No activity was found in control cells (without cytochrome P450 3A4) and their microsomal fractions, and the parent compound was unchanged after incubation. No metabolism was observed in the microsomal fraction of cells expressing cytochrome P450 3A4 without addition of NADPH

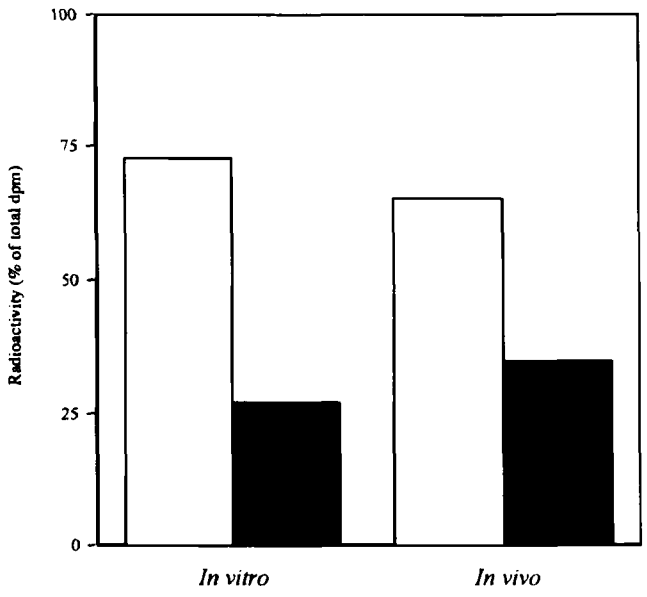

Fig. 4. In-vitro and in-vivo biotransformation of vamidothion by human cytochrome P450 3A4. (■) Vamidothion. (□) Vamidothion sulfoxide 10^4 dpm metabolic extracts from both whole cells and microsomal fractions of yeast expressing cytochrome P450 3A4 and control yeast were spotted on TLC plates. TLC plates were developed, metabolites were identified, excised from the TLC plates and quantified by scintillation counting. Activity is presented as percentage of total radioactivity (dpm) recovered.

(data not shown), further supporting the involvement of this monooxygenase in the biotransformation of vamidothion.

These studies show the value of yeast expression systems for investigating the metabolism of pesticides by individual P450s from mammalian tissues. Further studies should reveal the catalytic versatility of P450 3A4, which is likely to include many common pesticide substrates, judging by the number of drugs and carcinogens already studied. The enzyme may also have potential for application in bioremediation or herbicide tolerance strategies.

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Resistance to Antifungal Azoles in *Ustilago maydis* through Alteration of Sterol $\Delta^{5(6)}$ Desaturase

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Azole antifungals and their derivatives form a major group of antifungal compounds and have been shown to inhibit a P450 enzyme, sterol 14α-demethylase ($P450_{14\alpha\text{-}dm}$).[1] This inhibition results in the accumulation of 14α-methyl sterols such as lanosterol, obtusifoliol and 14α-methyl-3,6-diol (Fig. 1; for review, see Ref. 2) at the expense of the desmethyl sterols, most notably with a marked reduction in the levels of ergosterol (the principal sterol of most fungi). It is this ergosterol level reduction, coupled with the absence of alternative sterols able to support fungal growth,[3] that results in the primary antifungal effects of these agents.

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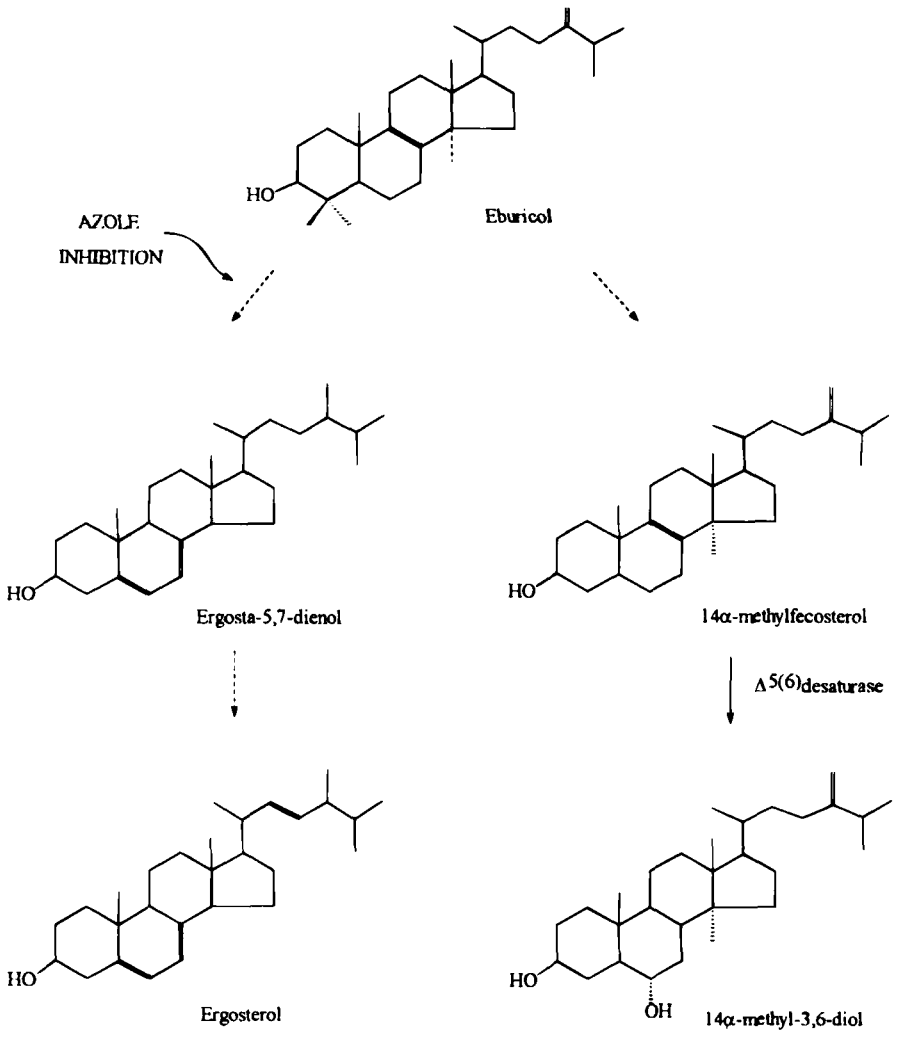

Fig. 1. Sterol biosynthetic pathway of *S. cerevisiae*, indicating the effect of azole inhibition of $P450_{14\alpha\text{-dm}}$.

The increasing incidence of resistance to antifungal azoles has become a significant problem in the treatment of patients suffering from AIDS, where fungal infection is both common and persistent in nature,[4] as well as in agriculture.[5] However, the underlying mechanisms of resistance remain unclear except in *Saccharomyces cerevisiae* Meyer ex Hanson, where resistance has been associated with a lesion in the sterol $\Delta^{5(6)}$ desaturase.[6] This mutation results in an altered sterol profile as a result of azole treatment, with accumulation of 14α-methylfecosterol instead of 14α-methyl-3,6-diol which accumulates when an active $\Delta^{5(6)}$ desaturase is present.[7] It is the presence of high levels of 14α-methylfecosterol in the *S. cerevisiae* mutants which enables growth to continue. The ability of a lesion in $\Delta^{5(6)}$ desaturase to confer resistance to azoles on inhibition of sterol 14α-demethylase is also reflected in the presence of sterol $\Delta^{5(6)}$ desaturase mutations as suppressors, allowing growth in mutant strains containing a gene disruption of sterol 14α-demethylase.[8] The degree of azole resistance of such strains is not increased by the presence of this second defect in the $P450_{14\alpha\text{-dm}}$, and suggests that this might also be true for other azole-resistant mutants blocked in $P450_{14\alpha\text{-dm}}$.[9]

Other alternative mechanisms of azole resistance have been proposed with varying levels of experimental support, including systems involving alteration in the efflux and/or influx[10] of these antifungal agents as well as altered cellular azole distribution.[11] As described above, $P450_{14\alpha\text{-dm}}$ mutants have also been isolated and found to be resistant to azoles. A common approach for the isolation of mutants containing lesions in the sterol biosynthetic pathway has been the screening of fungal populations with the polyene antibiotics. These compounds act through binding to ergosterol in the fungal membrane, promoting the loss of membrane integrity and ultimately resulting in growth arrest.[12] This approach in fungal pathogens has led to the isolation of some mutants with lesions in the $P450_{14\alpha\text{-dm}}$; for example the azole-resistant mutant of *Ustilago maydis* (DC) Corda, erg 40, which accumulates 14α-methylfecosterol.[13] However, as observed for sterol 14α-demethylase mutants of *S. cerevisiae*,[9] this strain may also be defective in sterol $\Delta^{5(6)}$ desaturase. Such double

TABLE 1

Sterol Profiles of *Ustilago maydis* (a) Wild-Type Strain ATCC 14826 and (b) Resistant Strain B3 after Treatment with Triadimenol[a]

	Sterol composition (%) at hours after treatment							*Untreated control*
Sterol	*0*	*3*	*6*	*9*	*12*	*18*	*24*	*24*
(a) Strain ATCC 14826								
Ergosta-tetraenol	3·6	—	—	1·8	2·0	—	—	2·3
Ergosta-8,22-dienol	0·6	—	—	—	—	—	—	2·7
Ergosterol	51·6	30·6	28·6	18·2	12·9	10·7	6·0	55·6
Ergosta-7,22-dienol	—	—	—	—	—	—	—	4·2
14α-Methyl fecosterol	3·2	—	—	11·8	12·3	11·6	17·8	—
Ergosta-5,7-dienol	27·4	4·9	11·2	7·8	5·1	5·5	3·1	28·2
Ergosta-7-enol	5·6	—	—	0·2	—	—	2·5	2·3
Obtusifoliol	—	14·6	11·9	25·9	32·4	23·8	33·4	0·5
14α-Methyl-3,6-diol[b]	—	—	—	—	—	5·6	5·8	0·5
Eburicol	1·9	43·7	42·4	31·2	34·0	36·6	29·3	1·9
Unidentified	6·1	6·2	5·9	3·1	1·3	6·2	2·1	1·1
Cell Counts ($ml^{-1} \times 10^{-5}$)	5·8(±0·1)	6·5(±0·1)	6·8(±0·1)	7·0(±0·1)	7·2(±0·1)	7·2(±0·1)	7·2(±0·1)	820(±14)
(b) strain B3								
Ergosta-tetraenol	4·9	trace	trace	—	—	—	—	2·7
Ergosterol	47·2	23·4	14·5	6·6	5·2	3·4	2·7	41·5
Ergosta-7,22-dienol	21·0	—	—	—	—	—	—	20·8
14α-Methylfecosterol	—	3·6	7·1	9·2	12·7	23·6	32·6	—
Ergosta-5,7-dienol	5·2	—	—	—	—	—	—	17·1
Ergosta-7-enol	10·7	—	—	—	—	—	—	3·9
4-Methylergosta-8,24-dienol	2·5	—	—	—	—	—	—	1·1
Obtusifoliol	—	6·3	17·7	18·4	21·7	24·1	24·6	—
Eburicol	3·9	66·7	60·7	65·8	60·5	41·9	31·0	1·7
Lanosterol	4·5	—	—	—	—	—	—	10·0
14α-methyl-3,6-diol[b]	—	—	—	—	—	2·6	4·7	—
Unidentified	—	—	—	—	—	4·4	4·4	8·4
Cell counts ($ml^{-1} \times 10^{-5}$)	5·0(±0·1)	17·5(±0·5)	35·0(±0·2)	60(±0·1)	75(±0·1)	190(±0·1)	480(±0·1)	540(±0·2)

[a] At 1×10^{-6} M, the MIC for strain ATCC 14826 treated with triadimenol. Sporidia (10^6 ml^{-1}) were inoculated into YEPD liquid medium (Difco peptone + glucose + Difco yeast extract, 20 + 20 + 10 g in water (1 litre)), and triadimenol was added and samples (100 ml) were removed at intervals for sterol extraction, cell counts and dry cell weight determination. Non-saponifiable sterols were extracted by the method of Woods[16] after sporidia were treated with potassium hydroxide and pyrogallol in methanol (90°C; 1 h). Extraction was completed using hexane (3 × 5 ml), the solvent being removed under nitrogen. The residue was silylated with BSTFA (20 μl) in toluene (100 μl; 1 h; 60°C). Sterols were analysed by GC-MS (VG 12-250 (VG BIOTECH)) using split injection with a split ratio of 20:1 and identified by reference to relative retention times and mass spectral data in the literature.[17–20]

[b] = 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

mutants have not been observed in any direct selection for azole resistance.

We have reported previously results for *U. maydis* demonstrating the general relevance of the azole-resistance mechanism established for *S. cerevisiae*.[14] In this summary we add to the previous observations which indicate that further examples of this type of mutation may occur in other resistant fungal pathogens such as *Candida albicans* (Robin) Berkhout and *Aspergillus fumigatus* (Fres.) which normally accumulate 14α-methyl-3,6-diol under treatment (for a review, see Ref. 2).

The mechanisms of azole-induced growth arrest have been characterised in *S. cerevisiae*, with arrest associated with the accumulation of 14α-methyl-3,6-diol (the probable product of attempted 5(6)-desaturation of 14α-methylfecosterol).[2] In *U. maydis* ATCC 14826, the involvement of this diol in growth arrest was considered unlikely as its accumulation occurred after the time when growth was arrested and then only to a relatively low concentration (Table 1a) when compared to that previously observed for *S. cerevisiae*.

The direct screening of a population of wild type *U. maydis* strain ATCC 14826, at a triadimenol concentration five-fold greater than the wild-type dose for minimal inhibition (MIC), resulted in the isolation of an azole-resistant mutant, termed B3. This mutant had a different sterol phenotype from its parent, with high levels of the Δ7 sterols (10·7% ergosta-7-enol and 21·0% ergosta-7,22-dienol) (Table 2). Accumulation of these sterols is indicative of a lesion in the sterol $\Delta^{5(6)}$ desaturase enzyme. However, the presence of low concentrations of Δ^5 desaturated sterols, for example ergosterol, indicated partial activity for the desaturase enzyme.

Mutant B3 was further examined for cross-resistance to a series of other antifungal compounds, such as other

TABLE 2
Sterol Compositions of *Ustilago maydis* Strains ATCC 14826 and B3 24 Hours after Treatment with Triadimenol[a]

			Strain		
Triadimenol concentration	*ATCC 14826 Untreated*	*B3 1×10^{-6} M*	*ATCC 14826 Untreated*	*B3 1×10^{-6} M*	*B3 1×10^{-5} M*
Sterols					
Ergosta-tetraenol	2·3	—	4·9	—	—
Ergosta-8,22-dienol	2·7	—	—	—	—
Ergosterol	55·6	1·9	47·2	2·7	6·4
Ergosta-7,22-dienol	4·2	—	21·0	—	—
14α-Methylfecosterol	—	18·2	—	32·6	53·3
Ergosta-5,7-dienol	28·2	0·3	5·2	—	—
Ergosta-7-enol	2·3	1·9	10·7	—	—
4-Methylergosta-8,24-dienol	—	—	2·5	—	—
Obtusifoliol	0·5	38·3	—	24·6	15·7
Eburicol	1·9	23·5	3·9	33·9	18·4
Lanosterol	—	—	4·5	—	3·2
14α-methyl-3,6-diol[b]	—	14·2	—	7·3	3·0
Unidentified	1·6	0·1	0·1	8·4	0·0

[a] Determined in shake culture in YEPD liquid medium (25°C; 150 rev min^{-1}). Sterol analyses were performed on sporidia harvested 24 h after addition of triadimenol using the method outlined in the footnote to Table 1.
[b] 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

azoles, the pyrimidine fenarimol, amphotericin B and cycloheximide (Table 3). Resistance to all the sterol $P450_{14\alpha\text{-dm}}$ inhibitors was observed whilst parent-strain levels of sensitivity to amphotericin B and cycloheximide were retained.

In order to examine the effect of azole treatment on B3, a 24-h growth study was conducted using the azole triadimenol at the dose determined as the MIC for the parent strain (Table 1b). As with the parent strain, azole uptake and accumulation at the target site were rapid, as indicated by the accumulation of the $P450_{14\alpha\text{-dm}}$ substrate, eburicol (66·7% of sterol at 3 h). The decrease in the levels of desmethyl sterols at this time also reflected this. Further evidence for the reduced activity of the $\Delta^{5(6)}$ desaturase enzyme in B3 was provided by the accumulation with time of 14α-methylfecosterol, indicating poor conversion of this sterol to 14α-methyl-3,6-diol. After 24 h, the levels of the two sterols were 32·6% and 4·7%, respectively, compared to levels of 18·2% and 14·2% for the treated wild-type strain, ATCC 14826. The accumulation of other intermediate 14α-methyl sterols in B3 and the consequent decrease in desmethyl sterols, was similar to that seen in the treated wild-type strain, indicating the involvement of a single lesion in the pathway.

TABLE 3
Comparison of Minimum Inhibitory Concentrations for Antifungal Azoles, Amphotericin B and Cycloheximide Treatments of ATCC 14826 and B3[a]

	Minimum inhibitory concentration	
Compound (concentration)	*ATCC 14826*	*B3*
Prochloraz (μM)	10	120
Diclobutrazole (μM)	0·5	10
Triadimenol (μM)	1	50
Tebuconazole (μM)	0·1	1
Fenarimol (μM)	5	50
Amphotericin B (μg ml^{-1})	1·0	1·0
Cycloheximide (μg ml^{-1})	0·01	0·01

[a] Determined in shake culture (150 rev min^{-1}); cell counts and colony forming units were assessed after three days. The azole antifungals were supplied as technical grade materials by their respective manufacturers. Stock solutions (10^{-4} M) were prepared in methanol (fenarimol, tebuconazole and procloraz), dimethyl sulfoxide (DMSO) (triadimenol, diclobutrazol) or at 1 mg ml^{-1} in DMSO (amphotericin B and cycloheximide).

Although the growth rate of B3 was unaltered in untreated culture when compared to ATCC 14826,[14] when B3 was treated with triadimenol its growth slowed down between the ninth and twelfth hours after treatment, at which stage the growth of ATCC 14826 had stopped (Tables 1a, b). At this stage, ergosterol and 14α-methylfecosterol concentrations were low (6·6% and 5·2% for B3, 9·2% and 12·7% for ATCC 14826). With ATCC 14826 treated with tradimenol at the MIC, growth arrest occurred once the combined proportions of these two sterols fell below 20%. By contrast, as time increased past this point, the growth rate of B3 increased, when 14α-methylfecosterol accumulated to levels >20% of total sterols.

Although B3 was resistant to azole compounds in comparative studies with ATCC 14826 involving concentrations at which growth of the latter was inhibited, growth of B3 was inhibited at higher concentrations.

Triadimenol treatment of B3 at its MIC, rather than that for ATCC 14826, resulted in a sterol profile consisting of 93·6% 14α-methyl sterols of which 53·3% represented 14α-methylfecosterol (Table 2). The reason for the accumulation of higher levels of 14α-methylfecosterol at the higher triazole dose is unclear, but it may result from a greater, and more rapid, accumulation of triadimenol at the target site. The level of 14α-methylfecosterol observed in B3 with this treatment might be expected to suport fungal growth and this implies a secondary antifungal mode of action of azole at this higher dose.

The combined sterol phenotype and spontaneous mutation frequency (10^{-8}) observed for the mutant B3 is consistent with a single mutation conferring resistance to azoles. In general this study confirms the overall relevance of the framework of mode of action and resistance established for *S. cerevisiae*, where a similar leaky $\Delta^{5(6)}$ desaturase mutant has been isolated.[7] Research conducted on *S. cerevisiae* dealing with this mechanism of resistance has indicated that many of the sterol $\Delta^{5(6)}$ desaturase mutants isolated had stringent blocks, but recent gene disruption studies have indicated that active sterol $\Delta^{5(6)}$ desaturase was required for aerobic growth.[15] Whether mutants completely blocked in sterol $\Delta^{5(6)}$ desaturase could be isolated in petite negative fungi remains to be determined and it may only be possible to isolate leaky sterol $\Delta^{5(6)}$ desaturase mutants.

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Role of Sterol $\Delta^{5,6}$ Desaturase in Azole Antifungal Mode of Action and Resistance

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In the last two decades extensive studies have been made to elucidate the mode of action of antifungal azole

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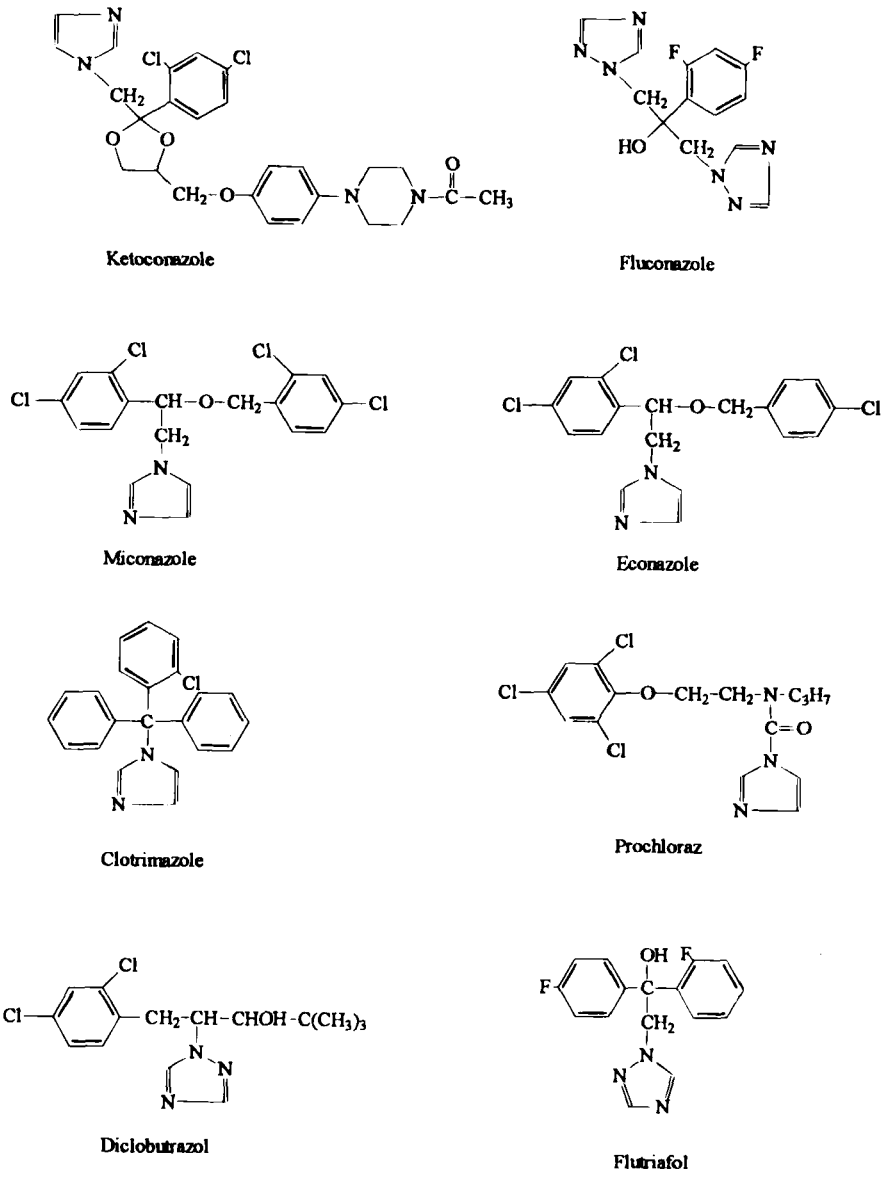

Fig. 1. The structures of the azole antifungal compounds used in this study.

compounds (Fig. 1). Studies have revealed that these compounds inhibit sterol 14α-demethylation, an important step of the ergosterol biosynthetic pathway of fungi, by direct interaction with the cytochrome P450 enzyme mediating this reaction. This causes decreased levels of ergosterol with a concurrent increase in 14α-methylated sterols.[1] In *Saccharomyces cerevisiae* Meyer ex Hansen azoles bind to microsomal P450(s)[2] and it has been shown that they inhibit lanosterol 14α-demethylation by a reconstituted system consisting of NADPH-cytochrome P450 reductase and the P450 involved in the 14α-demethylation of lanosterol.[3] However, the precise molecular details of cell-growth arrest, azole tolerance and azole resistance remain unclear.

Studies on azole-resistant mutants of *S. cerevisiae* have been valuable in studying resistance, including those with sterol 14α-demethylase mutants.[4,5] It has been shown previously that isolates resistant to fluconazole and ketoconazole were also defective in $\Delta^{5,6}$ desaturase[6,7] and it was concluded that inhibition of sterol 14α-demethylase with an azole could be overcome when the cell is defective in $\Delta^{5,6}$ desaturase. Subsequent investigation of sterols from azole-treated parental and mutant strains indicated a qualitative difference which enabled the important sterol conversion, resulting in cell growth arrest to be identified. The wild-type parental strain of *S. cerevisiae* accumulated lanosterol and 14α-methylergosta-8,24(28)-dien-3β,6α-diol (14α-methyl-3,6-diol), while a stringent $\Delta^{5,6}$ desaturase mutant accumulated lanosterol and 14α-methylergosta-8,24(28)-dien-3β-ol (14-methylfecosterol). It was concluded from these studies that sterol $\Delta^{5,6}$ desaturase activity is necessary for the formation of 14α-methyl-3,6-diol from 14α-methylfecosterol. The resistant strain was capable of growth on 14α-methylfecosterol and retention of the

14α group is not sufficient alone to inhibit cell growth except when 14α-methyl-3,6-diol is produced.

Studies on sterol 14α-demethylated gene-disrupted strains also indicate that they can grow only when they contain a second mutation in sterol $\Delta^{5,6}$ desaturase.[8] As expected for proteins which are targets for antifungal compounds, gene disruption of *S. cerevisiae* sterol 14α-demethylase is lethal.[9] There is no potential for leakiness in these mutants and this indicates that 14α-methylfecosterol may satisfy all the requirements for sterol in yeast growth. It had been observed previously that different requirements existed, i.e. a bulk role for sterol in membrane architecture and a 'sparking' hormonal role in growth.[10] Strains disrupted in their sterol 14α-demethylase were used here to probe the mode of action and resistance to azole antifungals.[8,11,12]

S. cerevisiae strains DK2 and DK3, which contain a gene-disrupted sterol 14α-demethylase, were used in this study. DK2 also contains a defective sterol $\Delta^{5,6}$ desaturase which suppresses the lethal effect of the sterol 14α-demethylase disruption. Analysis of sterol composition of the *S. cerevisiae* mutant DK3 following transfer from anaerobic to aerobic growth showed that ergosterol content rapidly declined during the period of growth arrest over 9 h, with a concurrent increase in the level of 14α-methyl-3,6-diol to over 80% of total cell sterol content (Table 1).

Transformation of the strains using the vector pW91P was carried out as described previously.[8] pW91P consists of the *Candida albicans* (Robin) Berkhout sterol 14α-demethylase gene with its own terminator and the *S. cerevisiae* phosphoglycerate kinase promoter cloned into the episomal vector, pEMBLYe30.[11] Expression of *C. albicans* sterol 14α-demethylase in the sterol 14α-demethylase-disrupted strain DK3 allowed aerobic growth, accumulating ergosterol (75%), 14α-methylfecosterol (11%), zymosterol (7%) and ergosta-5,7-dienol (5%). Complementation of DK2, containing defects in both sterol 14α-demethylase and sterol $\Delta^{5,6}$ desaturase, with the same expression construct caused accumulation of Δ^{7} sterols to 89% of the total cell sterol, ergosta-7,22-dienol (44%) and ergosta-7-enol (45%), instead of retaining 14α-methylfecosterol (75%) and lanosterol (14%), as observed in control transformants containing the empty vector.

Comparison of minimum inhibitory concentration (MIC) to antifungal azole compounds showed the DK2 transformants to be approximately ten-fold resistant to a range of such compounds compared to the DK3 transformants (Table 2) with 14α-methyl-3,6-diol accumulating in the latter as a result of treatment with azole. However, no difference was observed for the MIC of the sterol 14α-demethylase-defective strain DK2, with or without complementation.

Discussion

The effects of gene disruption are analogous to those with inhibition of sterol 14α-demethylase by azoles, and

TABLE 1

Growth Data and Sterol Composition for *Saccharomyces cerevisiae* Strain DK3 following Transfer (Time 0 h) from Anaerobic to Aerobic Growth Conditions[a,b,c]

Time (*h*)	*Growth* (*cells* ml^{-1}) × 10^7	*Sterol composition (%)*	
		Ergosterol	*14α-methyl-3,6-diol*[d]
0	1	75	0
3	3	35	35
6	8	15	59
9[e]	20	3	78

[a] Sterols were extracted and analysed as described in Ref. 18.

[b] Untransformed DK3 was grown anaerobically using Anaerocult reagents (BDH, UK) supplemented with ergosterol (20 μg ml^{-1}) from a stock solution in 'Tween' 80 surfactant + ethanol (1 + 1 by volume).

[c] For examination of sterol content during the period of growth arrest after transfer of DK3 to aerobic conditions, cells in active growth (1 × 10^7 cells ml^{-1}) were inoculated into YEPD liquid medium[9] containing glucose (100 g $litre^{-1}$).

[d] 14α-methylergosta-8,24(28)-dien-3β,6α-diol.

[e] Data for growth and sterol composition did not alter significantly over the period 9–24 h.

TABLE 2

Minimum Inhibitory Concentrations (MIC) μM for Antifungal Azole Compounds Applied to *Saccharomyces cerevisiae* Transformants[a,b]

Azole compound[c]	*Transformant*		
	pYe30 in DK2	*pW91P in DK2*	*pW91P in DW3*
Ketoconazole	2	2	0·2
Diclobutrazol	5	5	0·5
Econazole	100	100	20
Flutriafol	20	20	4
Fluconazole	500	500	20
Miconazole	1	1	0·2
Prochloraz	50	50	2
Clotrimazole	100	100	10

[a] In shake culture (30°C; 150 rev min^{-1}).

[b] Stationary growth-phase cells from plate cultures were resuspended (5 × 10^3 cells ml^{-1}) in yeast minimal medium containing yeast nitrogen base minus amino acids + glucose + histidine + tryptophan (6.7, 10 g $litre^{-1}$, 100 and 100 μg ml^{-1}, respectively). The test compound was added in dimethyl sulphoxide (DMSO) and the culture incubated on an orbital shaker (30°C: 150 rev min^{-1}; three days). Control cultures containing DMSO without azole were included for comparison. MIC values were constant over ≧ three replicate experiments.

[c] See Fig. 1.

consequently were used to investigate antifungal azole mode of action. Growth arrest of DK3 occurred when levels of ergosterol and 14α-methyl-3,6-diol were at a minimum and maximum, respectively. The accumulation of a sterol molecule with two hydroxyl groups in the *S. cerevisiae* cell membranes can be envisaged as having deleterious effects. The 6α-hydroxyl group is postulated to disrupt the normal hydrogen bonding between the sterol 3β-hydroxyl group with the carbonyl oxygen of the phospholipid-acyl chain.[13] Examination of suppressor mutants which could overcome the effects of the disruption showed them to be defective in sterol $\Delta^{5,6}$ desaturase, causing 14α-methylfecosterol to accumulate to 70–80% of the total cell sterol in place of 14α-methyl-3,6-diol. Hence, it was concluded that sterol $\Delta^{5,6}$ desaturase has a critical role in the mode of action of azole antifungal compounds, being involved in the formation of 14α-methyl-3,6-diol from 14α-methylfecosterol, accumulation of the former sterol being ultimately responsible for cell growth arrest with the concomitant depletion of ergosterol (Fig. 2).

To elucidate the contribution of both the sterol 14α-demethylase and sterol $\Delta^{5,6}$ desaturase defects to azole antifungal resistance, the sterol 14α-demethylase defect was complemented by heterologous expression of the same enzyme from *C. albicans*. Resistance could be attributable solely to the defect in sterol $\Delta^{5,6}$ desaturase, which prevented the formation of 14α-methyl-3,6-diol and caused production of 14α-methylfecosterol instead. The cells maintained growth under these conditions using 14α-methylfecosterol, albeit poorly (cell division time of approximately 4 h). These results reinforce the previous observations of Watson *et al.*[6] of the importance of 14α-methyl-3,6-diol in causing azole-induced cell growth arrest in *S. cerevisiae*. The results further challenge the proposed role of sterol function in *S. cerevisiae* with a 'sparking' requirement for trace levels of ergosterol in the organism.[14]

Previous research elucidated the sterol structural features required for viability by using strains auxotrophic for both sterol and haem biosynthesis.[10,15] Those studies revealed a sterol 'sparking' role where amounts of 0·01–0·1 μg ml^{-1} of a sterol in conjunction with larger amounts (5–20 μg ml^{-1}) of a different 'bulking' sterol were required to facilitate growth. Neither 'sparking' nor 'bulking' sterol alone permits growth at those concentrations.[10] Furthermore, the C5–6 unsaturation was found to be a necessary structure for 'sparking' sterols.[15] However, Arthington *et al.*[16] examined the gene encoding $\Delta^{5,6}$-desaturase and found it not to be essential for cell growth and viability in a haem-competent *S. cerevisiae* strain. Smith and Parks[17] resolved these differences by construction of three insertionally-inactivated $\Delta^{5,6}$-desaturase alleles, deleted in the amino-terminal, carboxy-terminal, or the entire encoding sequence, respectively. No ergosterol was detected in these strains and episterol was predominant,

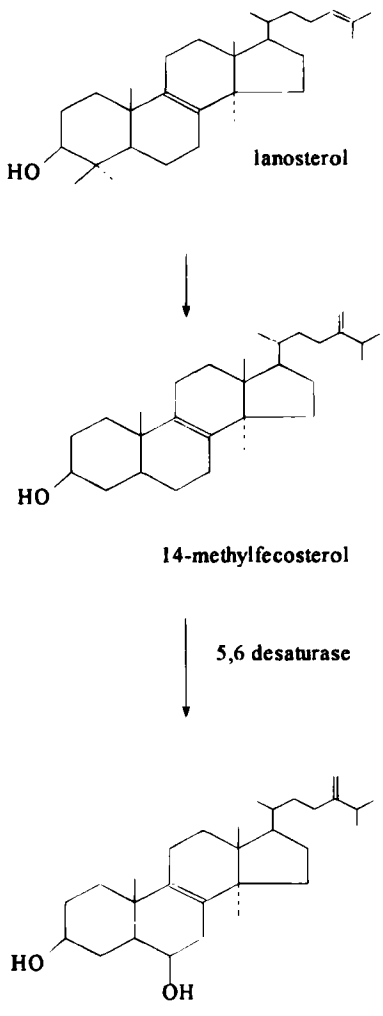

Fig. 2. The role of sterol $\Delta^{5,6}$-desaturase in the formation of 14α-methylergosta-8,24(28)-dien-3β,6α-diol (14-methyl-3,6-diol).

indicative of a lack of $\Delta^{5,6}$-desaturation. Smith and Parks[17] observed that gene disruption for sterol $\Delta^{5,6}$-desaturation was indeed not lethal in wild-type strains, but was lethal when using haem-deficient strains, as were employed in the initial experiments defining the 'sparking' role. This showed the 'sparking' role was relevant to haem-deficient strains. However, the same authors also showed that the gene-disrupted strains were capable of growth when glucose, but not glycerol, was used as a carbon source. This indicated that the mitochondria, required for aerobic growth, may be particularly sensitive to perturbations of sterol biosynthesis as was observed previously during the potent induction of petite mutations by antifungal azole.[5]

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